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PATENT TRADEMARK OFFICE

Docket No: 4058/1E827US1



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Frances H. ARNOLD; Huyn JOO

Serial No.: 09/246,451 Art Unit: 1652

Confirmation No.: 6181

Filed: February 9, 1999 Examiner: Manjunath N. RAO

For: OXYGENASE ENZYMES AND SCREENING METHOD

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**MARKED-UP COPY OF AMENDED CLAIMS FOR RESPONSE TO MAY 22, 2002 OFFICE ACTION**

Please amend the claims pursuant to 37 C.F.R. 1.121 as follows:

146. (Amended) A functional cytochrome P450 oxygenase variant comprising a mutation of a glutamic acid residue at a position corresponding to amino acid 331 of cytochrome $P450_{cam}$ from *P. putida* (SEQ ID NO:2) and having at least 90% sequence identity to SEQ ID NO:2.

147. (Amended) The cytochrome P450 oxygenase variant of claim 146,

wherein the mutant amino acid at the position corresponding to amino acid 331 is lysine.

148. (Amended) A functional cytochrome P450 oxygenase variant comprising at least one mutation of an arginine residue at a position corresponding to amino acid 280 of cytochrome $P450_{cam}$ from *P. putida* (SEQ ID NO:2) and having at least 90% sequence identity to SEQ ID NO:2.

149. (Amended) The cytochrome P450 oxygenase variant of claim 148, wherein the mutant amino acid at the position corresponding to amino acid 280 is leucine.

150. (Amended) A functional cytochrome P450 oxygenase variant comprising at least one mutation of a cysteine residue at a position corresponding to amino acid 242 of cytochrome $P450_{cam}$ from *P. putida* (SEQ ID NO:2) and having at least 90% sequence identity to SEQ ID NO:2.

151. (Amended) The cytochrome P450 oxygenase variant of claim 148, wherein the mutant amino acid at the position corresponding to amino acid 280 is phenylalanine.

152. (Amended) A functional cytochrome P450 oxygenase variant comprising at least one mutation at a position selected from the group consisting of amino acid positions 242, 280, and 331 of SEQ ID NO:2 and having at least 90% sequence identity to SEQ ID NO:2.

153. (Amended) The cytochrome P450 oxygenase variant of claim 152 comprising at least one mutation selected from the group consisting of:

(a) a glutamic acid to lysine mutation at position 331 of SEQ ID NO:2;

(b) an arginine to leucine mutation at position 280 of SEQ ID NO:2; and

(c) a cysteine to phenylalanine mutation at position 242 of SEQ ID NO:2.

154. (Amended) [A function-conservative variant of the] The variant cytochrome P450 oxygenase of claim [153] 152, comprising at least one mutation selected from the group consisting of:

(a) a glutamic acid to arginine or histidine mutation at position 331 of SEQ ID NO:2; and

(b) an arginine to isoleucine, methionine, or valine mutation at position 280 of SEQ ID NO:2.

155. (Amended) [An] A functional oxygenase enzyme variant encoded by a first polynucleotide that hybridizes to a second polynucleotide under conditions of high

stringency, which second polynucleotide encodes the cytochrome P450 oxygenase enzyme of claim 153.

156. (Amended) [An evolved] A cytochrome P450 oxygenase variant having a catalytic activity at least two times the catalytic activity of wild-type cytochrome $P450_{cam}$ oxygenase from *P. putida* (SEQ ID NO:2) in promoting the oxygenation of an oxygenase substrate in the presence of an oxygen donor and at least 90% sequence identity to SEQ ID NO:2.

157. (Amended) [An evolved] A cytochrome P450 oxygenase variant having a catalytic activity at least ten times the catalytic activity of wild-type cytochrome $P450_{cam}$ oxygenase from *P. putida* (SEQ ID NO:2) in promoting the oxygenation of an oxygenase substrate in the presence of an oxygen donor and at least 90% sequence identity to SEQ ID NO:2.

158. (Amended) [An evolved] A cytochrome P450 oxygenase variant having a stability at least two times the stability of wild-type cytochrome $P450_{cam}$ oxygenase from *P. putida* (SEQ ID NO:2) in promoting the oxygenation of an oxygenase substrate in the presence of an oxygen donor and at least 90% sequence identity to SEQ ID NO:2.

159. (Amended) [An evolved] A cytochrome P450 oxygenase variant having a stability at least ten times the stability of wild-type cytochrome $P450_{cam}$ oxygenase from *P. putida* (SEQ ID NO:2) in promoting the oxygenation of an oxygenase substrate in the presence of an oxygen donor and at least 90% sequence identity to SEQ ID NO:2.

161. (Amended) An oxygenase variant evolved from a wild-type oxygenase enzyme, and having a catalytic activity at least ten times the catalytic activity of the wild-type oxygenase enzyme in promoting the oxygenation of an oxygenase substrate in the presence of an oxygen donor and at least 90% sequence identity to SEQ ID NO:2, which oxygenase variant was identified by a method comprising the steps of:

(a) contacting a test enzyme variant with an oxygenase substrate and [an] the oxygen donor under conditions allowing the formation of an oxygenated product if said test enzyme variant is an oxygenase enzyme; [and]

(b) providing a coupling enzyme which is capable of promoting the formation of a detectable composition from the oxygenated product; [and]

(c) detecting the detectable composition; and

(d) selecting any test enzyme having at least 10 times the catalytic activity of the wild-type oxygenase enzyme in the presence of the oxygen donor and at least 90% sequence identity to SEQ ID NO:2.

163. (Amended) The oxygenase variant of claim 161, wherein

(a) the organic substrate is selected from the group consisting of naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, anthracene, benzphetamine, and coumarin;

(b) the oxygen donor is selected from the group consisting of hydrogen peroxide and t-butyl peroxide; and

(c) the coupling enzyme is selected from the group consisting of horseradish peroxidase, cytochrome c peroxidase, tulip peroxidase, lignin peroxidase, carrot peroxidase, peanut peroxidase, soybean peroxidase, and [peroxidase Novozyme®] NOVOZYME® 502.

164. (Amended) An oxygenase variant evolved from a wild-type oxygenase enzyme, and having a stability at least ten times the stability of the wild-type oxygenase enzyme in promoting the oxygenation of an oxygenase substrate in the presence of an oxygen donor and at least 90% sequence identity to SEQ ID NO:2, which oxygenase variant was identified by a method comprising the steps of:

(a) contacting a test enzyme variant with an oxygenase substrate and [an] the oxygen donor under conditions allowing the formation of an oxygenated product if said test enzyme variant is an oxygenase enzyme; [and]

(b) providing a coupling enzyme which is capable of promoting the formation of a detectable composition from the oxygenated product; [and]

(c) detecting the detectable composition; and

(d) selecting any test enzyme having at least 10 times the stability of the wild-type oxygenase enzyme in the presence of the oxygen donor and at least 90% sequence identity to SEQ ID NO:2.

166. (Amended) The oxygenase variant of claim 164, wherein

(a) the organic substrate is selected from the group consisting of naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, anthracene, benzphetamine, and coumarin;

(b) the oxygen donor is selected from the group consisting of hydrogen peroxide and t-butyl peroxide; and

(c) the coupling enzyme is selected from the group consisting of horseradish peroxidase, cytochrome c peroxidase, tulip peroxidase, lignin peroxidase, carrot peroxidase, peanut peroxidase, soybean peroxidase, and [peroxidase Novozyme®] NOVOZYME® 502.

167. [An] A functional cytochrome P450 oxygenase variant comprising a mutation at a position corresponding to at least one of amino acid 331, 280, and 242 of cytochrome $P450_{cam}$ from *P. putida* (SEQ ID NO:2) and having at least 90% sequence identity to SEQ ID NO:2, which cytochrome P450 oxygenase variant was identified by a method comprising the steps of:

(a) contacting a test cytochrome P450 oxygenase variant with an oxygenase substrate and an oxygen donor under conditions allowing the formation of an oxygenated product if said test enzyme variant is an oxygenase enzyme; [and]

(b) providing a coupling enzyme which is capable of promoting the formation of a detectable composition from the oxygenated product; [and]

(c) detecting the detectable composition; and

(d) selecting any test enzyme having a mutation at a position corresponding to at least one of amino acid 331, 280, and 242 of cytochrome $P450_{cam}$ from *P. putida* (SEQ ID NO:2) and at least 90% sequence identity to SEQ ID NO:2.

169. (Amended) The cytochrome P450 oxygenase variant of claim 167, wherein

(a) the organic substrate is selected from the group consisting of naphthalene, 3-phenylpropionate, benzene, toluene, benzoic acid, anthracene, benzphetamine, and coumarin;

(b) the oxygen donor is selected from the group consisting of hydrogen peroxide and t-butyl peroxide; and

(c) the coupling enzyme is selected from the group consisting of horseradish peroxidase, cytochrome c peroxidase, tulip peroxidase, lignin peroxidase, carrot peroxidase, peanut peroxidase, soybean peroxidase, and [peroxidase Novozyme®] NOVOZYME® 502.